EXPLORE THE ROLE OF PHARMACOLOGICAL INTERVENTION IN EXPERIMENTALLY INDUCED FATTY LIVER DISEASE

SYNOPSIS

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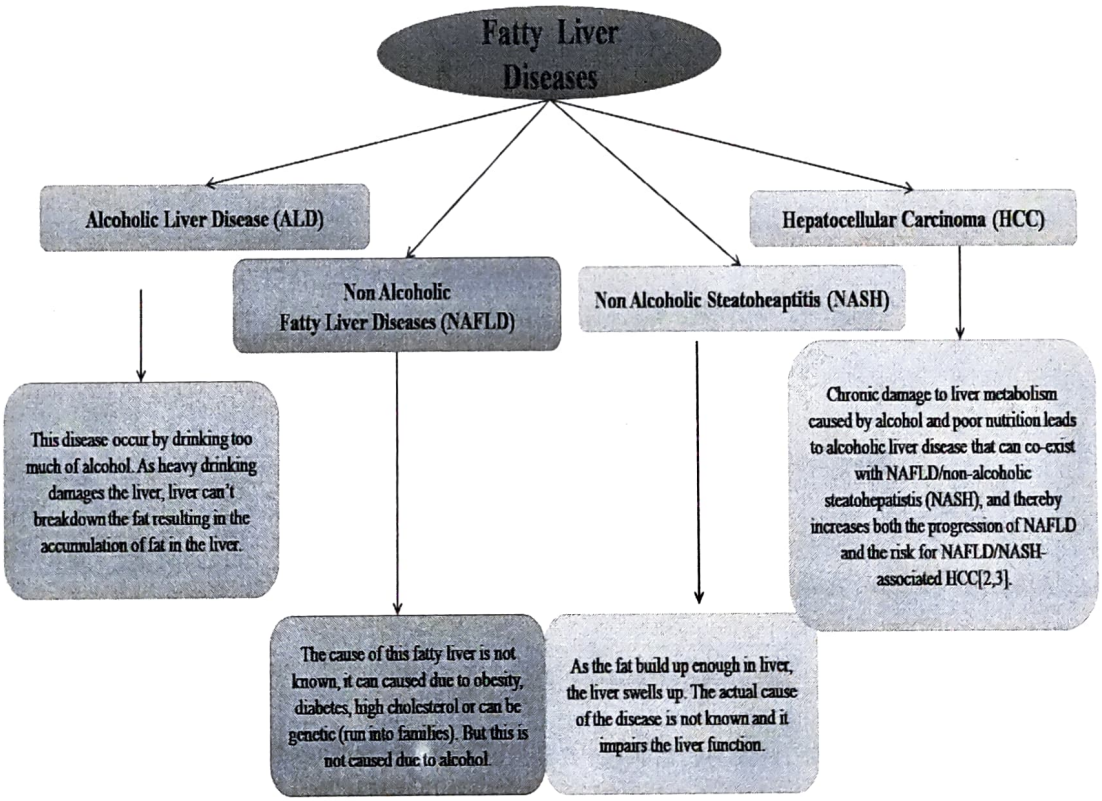
INTRODUCTION

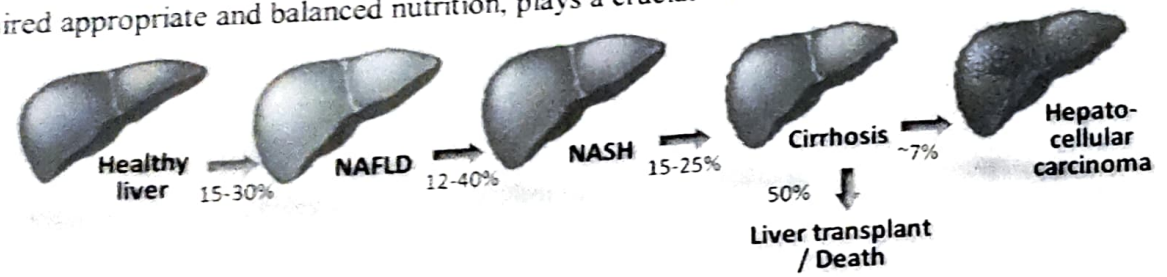
Disease Profile:

The liver is the largest glandular organ in the body and performs numerous vital functions to keep the body pure and out of toxins and harmful substances. The demolition of old red blood cells produces waste that gives facial matter usually in brown colour. Discoloration of stool or darkened urine could signal something is wrong with the liver. Another common sign of liver problems is jaundice, the yellowing of the skin and eyes due to the build-up of bilirubin, a waste product of normally haemoglobin collapse. Because the liver performs so many vital functions it is now and then prone to disease. Due to overweight and drinking too much alcohol can damage the liver. (Sankar, 2020)


Fatty Liver Diseases
Alcoholic Liver Disease (ALD)
Non Alcoholic Fatty Liver Diseases (NAFLD)
Non Alcoholic Steatoheaptitis (NASH)
Hepatocellular Carcinoma (HCC)
This disease occur by drinking too much of alcohol. As heavy drinking damages the liver, liver can't breakdown the fat resulting in the accumulation of fat in the liver.
The cause of this fatty liver is not known, it can caused due to obesity, diabetes, high cholesterol or can be genetic (run into families). But this is not caused due to alcohol.
As the fat build up enough in liver, the liver swells up. The actual cause of the disease is not known and it impairs the liver function.
Chronic damage to liver metabolism caused by alcohol and poor nutrition leads to alcoholic liver disease that can co-exist with NAFLD/non-alcoholic steatohepatistis (NASH), and thereby increases both the progression of NAFLD and the risk for NAFLD/NASH-associated HCC[2,3].

Fatty liver is a common problem seen in many urban parts of India it goes untreated till it changes into serious disease like hepatitis (jaundice). At its initial stage really harmless but it can slowly progresses to a probable slow killer. The Fatty liver disease was first named in 1980, after

studying number of obese or diabetic patients having excess fat in their lives. Since then, researchers ascertained that certain fat in the liver is healthy without facing a problem. Once the amount of fat reaches 5 to 10 % of the total weight of the liver which is the clear warning of fatty liver disease, it is much more prone to damage with swelling, scarring, and liver failure. Even Hough the accurate grounds of the disease is unknown, yet there is ample evidence that eating too much or unhealthy foods like highly-processed or highly-fat containing foods devoid of required appropriate and balanced nutrition, plays a crucial function.



This disease represents a wide spectrum of clinical entities ranging from asymptomatic hepatic steatosis to more advanced non- alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma and is commonly seen in patients with diabetes mellitus (DM) and met- abolic syndrome (MetS) (Alshehri *et al.*, 2021) (Anstee *et al.*, 2019). Currently, the global burden of the NAFLD on the health system is rapidly increasing with alarming prevalence and mortality rates(Asrani *et al.*, 2019). Recent epidemiological data have shown high prevalence close global rates with about 32% in the middle east, 30% in the USA and South America, 27% in Asia, 24% in Europe, and 13.1% in Africa (Mitra et al., 2020) (Younossi *et al.*, 2016). With no current approved therapies, managing the lifestyle, diet, and weight loss remain the available therapeutic intervention (Munteanu et al., 2016). Along with, some pharmacological therapies such as insulin sensitizers, incretin-based drugs,weight loss bills, lipid-lowering drugs, and bariatric surgery, as well in few cases liver transplantations are used in the man- agement of NAFLD (Diehl and Day, 2017) (Pappachan *et al.*, 2017)

Diet in alcoholic liver disease: The dietary recommendations are purely depend on the stage of the disease. The diet should be protein and calories rich so that the regeneration process is quicker. In advanced cirrhosis, it is advisable intake of foods that are moderate or low in proteins. In alcoholic liver disease, the person often has severe nutritional deficiency and malnourishment so that it is very essential to provide all the vitamins, nutrients, and minerals for

fast recovery and to prevent the harmful effects of alcohol on the metabolism. When the organ is affected, the amount of bile produced by the liver is not sufficient enough for the digestion of a high fat diet. Alcohol interferes with fatty acid metabolism, which leads to fat deposition in the liver. Hence, a low fat diet, rich in omega-3 fatty acids and less triglycerides, can decrease the severity of this disease. (Sankar, 2020)

Diet in modern days contain high processed foods, hydrogenated vegetable oils, especially sugar and carbohydrates have caused an epidemic of fatty liver disease and obesity. Exposure to excess alcohol, cigarettes and toxic chemicals also overloads the liver causing liver dysfunction. Alcoholic liver damage is seen among the productive age group people with high morbidity and mortality is connected with the matter of huge economic loss to the entire world and left the society with unending grief. Even though most people with NAFLD do not develop serious liver disease but it has become very common in recent years due to epidemic in obesity, NAFLD has become a common cause of cirrhosis. There needs to be more awareness of liver disease so that patients can be treated early to prevent, liver failure and cirrhosis (liver cancer). A lot has been done regarding the epidemiology, pathogenesis, diagnosis and treatment of patients with NAFLD. There are no successful forms of treatment available to cure the diseases. However, new developments in the therapeutic world and changed nutritional approaches have shown the way to better strategies in the management of this condition. However, there is every need for further prospective and cooperative studies and extensive investigations.(Sankar, 2020)

Drug Profile:

sodium orthovanadate (Na_3VO_4), a complex of vanadate with hydrogen peroxide, is an inorganic salt of vanadium and broadly used as an inhibitor of protein phosphatases [protein serine/threonine phosphatases (PS/TPs) and protein tyrosine phosphatases (PTPs)] with structural properties similar to phosphate. Vanadium-based compounds, including Na_3VO_4, are attracting attention for their insulin-mimetic effect. Na_3VO_4 inhibits the activity of PTP1B and moves the glucose transporter type 4 (GLUT4) channels to the cell membrane, ultimately increasing sugar uptake. In addition, administration of Na_3VO_4 lowers elevated blood sugar level in various diabetic rat models.

The role of PTP1B as a metabolic modulator has been extensively reported in preclinical models of diet-induced insulin resistance and obesity in mice with global or tissue-specific deletion of

the *Ptpn1* gene(Klaman *et al.*, 2000)(Delibegovic *et al.*, 2009) as well as in humans (Ahmad *et al.*, 1997). On the other hand, NAFLD is a chronic liver disease that, in its initial stages, is directly associated with fatty liver and hepatic insulin resistance, in which PTP1B plays also a major role (Delibegovic *et al.*, 2009) (González-Rodríguez *et al.*, 2018) (Zabolotny *et al.*, 2008). Despite this extensive knowledge, much less is known on the involvement of PTP1B during NAFLD progression, particularly in NASH, in which the inflammatory component is crucial for disease progression.

LITERATURE REVIEW:

Zhang *et al.*, 2021 studied pathogenesis of NAFLD and AFLD is linked to lipid buildup, oxidative stress, insulin resistance, inflammation, and dietary behaviours. The mechanism that increases illness development, on the other hand, is yet unknown. (Zhang *et al.*, 2021)

Johnston et al., 2020 AFLD is linked to excessive alcohol intake and is responsible for 47.9% of cirrhosis fatalities globally (Johnston et al., 2020).

Lian et al., 2020 upregulating SREBP1/2 and downregulating PPAR, CdCl2 enhances hepatic lipogenesis and suppresses fatty acid oxidation. Insulin resistance, dyslipidemia, and metabolic/cardiovascular disorders have all been linked to high fat consumption, which is typical of so-called Western diets. NAFLD progression is thought to occur when the adaptive mechanism designed to counteract FFA-induced lipotoxicity in the liver is overwhelmed, resulting in ER stress, autophagy disorder, gut microbiota imbalance, microRNA dysregulation, genetic/epigenetic risk factors, mitochondrial dysfunction, and an increase in inflammatory cytokines, among other things (Lian *et al.*, 2020).

Younossi et al., 2019 most common metabolic liver disorders are non-alcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease (AFLD). No particular medications have been approved yet because to the complicated pathogenic processes of NAFLD and AFLD. The pathogenesis of NAFLD and AFLD is linked to lipid buildup, oxidative stress, insulin resistance, inflammation, and dietary behaviours. The mechanism that increases illness development, on the other hand, is yet unknown. NAFLD affects almost a quarter of the world's adult population (Younossi *et al.*, 2019).

Shirpoor et al., 2018 PTP1B has recently been related to NAFLD in a rising series of researches, indicating that it is an important player in the progression of NAFLD through regulating hepatic

lipogenesis, insulin resistance, and endoplasmic reticulum stress (ERS). PTP1B appears to play a role in ALD, Ethanol leads to the upregulation of PTP1B in the muscle and liver of rats and muscle of macaque (Shirpoor *et al.*, 2018).

Benedict et al., 2017 Non-alcoholic fatty liver disease (NAFLD) encompasses the simple steatosis to more progressive steatosis with associated hepatitis, fibrosis, cirrhosis, and in some cases hepatocellular carcinoma. NAFLD is a growing epidemic, not only in the United States, but worldwide in part due to obesity and insulin resistance leading to liver accumulation of triglycerides and free fatty acids. Numerous risk factors for the development of NAFLD have been espoused with most having some form of metabolic derangement or insulin resistance at the core of its pathophysiology. NAFLD patients are at increased risk of liver-related as well as cardiovascular mortality, and NAFLD is rapidly becoming the leading indication for liver transplantation. Liver biopsy remains the gold standard for definitive diagnosis, but the development of noninvasive advanced imaging, biochemical and genetic tests will no doubt provide future clinicians with a great deal of information and opportunity for enhanced understanding of the pathogenesis and targeted treatment. As it currently stands several medications/ supplements are being used in the treatment of NAFLD; however, none seem to be the "magic bullet" in curtailing this growing problem yet. In this review we summarized the current knowledge of NAFLD epidemiology, risk factors, diagnosis, pathogenesis, pathologic changes, natural history, and treatment in order to aid in further understanding this disease and better managing NAFLD patients.(Benedict and Zhang, 2017)

Kanwar et al,. 2016 states that metabolic syndrome is a conglomerate of cardiovascular risk factors which predispose a person to developing type II diabetes and cardiovascular disease. The current diagnostic criteria require having 3 of 5 of the following factors: Triglycerides 150 mg/dL or greater, high-density lipoprotein-cholesterol of less than 40 mg/dL in men and less than 50 mg/dL in women, hyperglycemia (fasting glucose of 100 g/dL or greater), an increased waist circumference (defined by population specific data), and hypertension (systolic blood pressure of 130 mmHg or greater or diastolic blood pressure of 85 mmHg or greater). As previously mentioned the incidence of NAFLD has been increasing in concert with the rising rates of metabolic syndrome. In fact it has been stated that the incidence of NAFLD increases with increasing number of metabolic syndrome criteria met (Kanwar and Kowdley, 2016).

Lonardo et al., 2015 compared to non-diabetic patients (matched for age, sex, and body weight), type 2 diabetes mellitus (T2DM) patients have liver fat contents that are 80% higher . Interestingly, it has been shown that T2DM patients with NAFLD can have normal liver function tests, which may lead one to believe that the prevalence of NAFLD in T2DM patients is much higher than reported in this patient population (Lonardo *et al.*, 2015).

Sung et al., 2014 Additionally, T2DM patients display a very high risk of developing NASH as well as a two-to-four-fold increased risk of fatty liver associated complications (Sung, Wild and Byrne, 2014)

Lindtner et al 2013 binge drinking increases hypothalamic PTP1B and induces systemic insulin resistance in rats, which is prevented by the administration of a PTPB inhibitor (Lindtner *et al.*, 2013).

Polyzos et al. 2012 PTP1B has long been suspected of playing a role in metabolic disorders such as obesity and type 2 diabetes. PTP1B's physiological functions have recently been discovered to have a critical role in the pathology of liver damage. The underlying metabolic abnormalities indicated above exacerbate liver dysfunction and increase the risk of liver fibrosis in NAFLD patients. Despite the rising knowledge base in the study of NAFLD, the processes that support its development are still unknown (Polyzos et al. 2012).

Shimizu et al. 2003 & Shi et al. 2007 studied that PTP1B membrane localization is required for activation of SREBP1 expression and hepatic triglyceride production in hepatocytes. PTP1B overexpression in hepatocytes enhanced the content and transcriptional activity of SREBP1a and 1c, resulting in an increase in hepatic fatty-acid and triglyceride production. When PTP1B was over-expressed, the transcriptional activity of Sp1 sites in SREBP1 promoters was increased, which was attributable to increased activity of phosphatase 2A (PP2A). (Shimizu *et al.*, 2003) (Shi *et al.*, 2007).

Lieber et al. 1991 Alcohol metabolism takes place mostly in the liver and, to a lesser extent, the gastrointestinal system. Alcohol dehydrogenase and cytochrome P-450 2E1 are the two primary routes for alcohol metabolism in the liver (CYP2E1). Alcohol dehydrogenase (ADH) is a cytosolic enzyme found in hepatocytes that transforms ethanol to acetaldehyde. Acetaldehyde is then converted to acetate by the enzyme acetaldehyde dehydrogenase in the mitochondria (ALDH). The decrease of NAD to NADH is linked to both stages (Lieber et al. 1991)

Grunnet et al., 1986 studied that Increased NADH/NAD ratio has a significant impact on glucose and lipid metabolism. With acetyl-coA redirected to ketogenesis and fatty acid synthesis, gluconeogenesis is inhibited and substrate flow through the citric acid cycle is reduced. This latter consequence of the altered redox state, together with the suppression of mitochondrial fatty acid b-oxidation, leads to the pathophysiology of fatty liver disease (steatosis) (Grunnet and Kondrup, 1986).

Barford et al. 1994 studied that PTPs are quickly gaining traction as potential next-generation therapeutic targets. PTP1B (protein tyrosine phosphatase 1B) is the founding member of the PTP superfamily and represents the enzymes' non-transmembrane or cytosolic form (Barford et al. 1994).

RESEARCH GAP IDENTIFICATION

Fatty liver diseases like alcoholic and non alcoholic fatty liver disease involves progressive hepatic function and alteration due to diet modification, insulin resistance, oxidative stress and inflammation which leads to hepatocellular carcinoma. PTPase is a crucial player in the progression in alcoholic and non alcoholoic fatty liver disease by regulating the hepatic lipogenesis, insulin resistance and endoplasmic reticulum stress (ERS). Therefore we hypothesize that targeting protein tyrosine phosphatase with two different disease condition with their antagonist may preserve hepatic function and alteration.

OBJECTIVES

The aim of present investigation is to:

1. To determine the effect of selective intervention in rodent model of cadmium induced and High fat diet induced Non alcoholic fatty liver disease.
2. To determine the effect of selective intervention in rodent model of alcohol induced and High fat diet+alcohol induced alcoholic fatty liver disease.
3. To investigate the effect of pharmacological intervention in different disease condition of fatty liver disease.

PROPOSED METHODOLOGY

Materials:-

Chemicals and instruments:-

List of chemicals and kits used in study

Sr. No	Chemical name	Company
1.	Carboxy methyl cellulose	SRL Mumbai
2.	DTNB (5, 5- Dithiobis-2- nitro benzoic acid)	SRL Mumbai
3.	EDTA	Loba chemie
4.	Glutathione	Loba chemie
5.	Hydrochloric acid	SRL Mumbai
6.	Hydrogen peroxide	Loba chemie
7.	Hydroxyl amine HCL	SRL Mumbai
8.	Sodium arsenate	Loba chemie
9.	Sodium azide	Loba chemie
10.	Sodium lauryl sulphate	Loba chemie
11.	Thiobarbituric acid	Loba chemie
12.	Trichloroacetic acid	SRL Mumbai
	Drugs	
13.	Cadmium chloride	SDFCL
14.	Sodium Orthovenadate	SRL Mumbai
	Estimation kits	
15.	ALP	Span diagnostic
16.	LDH	Span diagnostic
17.	SGOT	Span diagnostic
18.	SGPT	Span diagnostic
19.	Total Bilrubin	Span diagnostic
20.	Total cholesterol	Span diagnostic
21.	Triglyceride	Span diagnostic
22.	Uric acid	Span diagnostic

Table: List of instruments used in the study:-

Instrument name	Company
Centrifuge	Remi Motors Ltd.
Chemical weighing balance	Shimadzu, Singapore
Auto analyser	Micro lab 300
Cooling centrifuge	Remi Electrotechnik Ltd.
Power Lab	AD Instruments, NSW, Australia
Deep freezer	Carrier Air conditioning & Refrigeration Ltd.
Tissue homogenizer	Remi Electrotechnik Ltd.
Animal weighing balance	Samson
Vortex	Spinix
UV/VIS double beam spectrophotometer	Shimadzu, Singapore
Hot air oven	Lyzer
Water bath	Perfit India
pH meter	Control dynamics
Mechanical Stirrer	Remi motors Ltd.

Animals used in experimental study:-

Adult Wistar rats (either sex), weighing between, will be procured from registered breeder. The animals were kept in quarantine section till monitoring of health status of animals and subsequently transferred to the housing area. The animals will be acclimatized for seven days to the housing conditions of central animal house facility. Animals will housed in polypropylene cages with dust free rice husk as a bedding material and maintained under standard laboratory conditions with controlled temperature (23 ± 2°C), humidity (40 ± 10%) and natural (12 hr each) light-dark cycle. The animals were fed with standard rodent pellet diet (Ashirwad Industries, Mohali) and water *ad libitum*. The care of laboratory animals was done following the guidelines of CPCSEA.

Experimental Protocol:

This study will carry out on wistar rat weighing approximately 150-200 g of either sex and will divide into following group:

Experimental Design (Grouping of animals):

Experiment protocol 1:

Group 1: Normal Control (n=6);

Group 2: Perse Control (n=6);

Group 3: Cadmium chloride (10mg/l for 8 weeks) induced NAFLD (n=08) (Alshehri et al., 2021);

Group 4: SOV low dose (5mg/kg/p.o) (n=08) (Joshi *et al.*, 2022)+ Cadmium chloride (10mg/l for 8 weeks);

Group 5: SOV high dose (10mg/kg/p.o) (n=08) (Joshi *et al.*, 2022) + Cadmium chloride (10mg/l for 8 weeks);

Group 6: Standard dose (n=8); all animals will be observed for 8 weeks.

Experiment protocol 2:

Group 1: Normal Control (n=6);

Group 2: Perse Control (n=6);

Group 3: HFD induced NAFLD (n=08); (Van *et al.*, 2017)

Group 4: SOV low dose (5mg/kg/p.o) (n=08) (Joshi *et al.*, 2022)+ HFD induced NAFLD;

Group 5: SOV high dose (10mg/kg/p.o) (n=08) (Joshi *et al.*, 2022) + HFD induced NAFLD;

Group 6: Standard drug (n=8); all animals will be observed for 8 weeks.

Experiment protocol 3:

Group 1: Normal Control (n=6);

Group 2: Perse Control (n=6);

Group 3: alcohol (10ml/kg for 8 weeks) induced AFLD (n=08) (Xu Wang et al., 2021);

Group 4: SOV low dose (5mg/kg/p.o) (n=08) (Joshi *et al.*, 2022) + alcohol (10ml/kg for 8 weeks) induced AFLD;

Group 5: SOV high dose (10mg/kg/p.o) (n=08) (Joshi *et al.*, 2022) + alcohol (10ml/kg for 8 weeks) induced AFLD;

Group 6: Standard dose (n=8) all animals will be observed for 8 weeks.

Experiment protocol 4:

Group 1: Normal Control (n=6);

Group 2: Perse Control (n=6);

Group 3: alcohol + HFD (10ml/kg for 8 weeks) induced AFLD (n=08) (Xu Wang et al., 2021);

Group 4: SOV low dose (5mg/kg/p.o) (n=08) (Joshi *et al.*, 2022) + alcohol (10ml/kg for 8 weeks) + HFD induced AFLD;

Group 5: SOV high dose (10mg/kg/p.o) (n=08) (Joshi *et al.*, 2022) + alcohol (10ml/kg for 8 weeks) + HFD induced AFLD;

Group 6: Standard dose (n=8) all animals will be observed for 8 weeks.

PARAMETERS: Pathological evaluation, biochemical parameters like SGOT, SGPT, GGT, histopathology, oxidative stress markers TBARS, GSH, SOD, GPx, marker of inflammation will be evaluated

Assessment of Serum Biomarkers:

The blood sample will be collected from the rats after completion of the study duration. The blood was collected by puncturing retro-orbital plexus using glass capillary tubes under light the suitable anaesthesia. At the end of experimental period the blood samples were taken and plasma/serum was separated for analysis of different haematological parameters alkaline Phosphatase (ALP), aspartate amino transferase (AST), alanine amino transferase (ALT), lactate

dehydrogenase (LDH), total bilirubin, cholesterol, uric acid, triglyceride. All analysis was performed with commercially available kits using autoanalyzer (Reckon Diagnostics, Chandigarh and Span Diagnotics Ltd., Surat, Erba Diagnostics., Baddi)

Estimation of Serum Glutamic Pyruvic transaminase (SGPT)

a) Principle:

Alanine aminotransferase catalyses the transamination of L- Alanine and α- ketoglutarate to form pyruvate and L- Glutamate. In subsequent reaction, Lactate Dehydrogenase (LDH) reduces Pyruvate to Lactate with simultaneous oxidation of Nicotinamide Adenine Dinucleotide (reduced) (NADH) to Nicotinamide Adenine Dinucleotide (NAD). The rate of oxidation of NADH is measured kinetically by monitoring the decrease in absorbance at 340 nm. LD rapidly and completely reduces endogenous sample Pyruvate during the initial incubation period, so that it does not interfere with the assay.

L- Alanine + 2- Oxoglutarate $\xrightarrow{ALT}$ Pyruvate + L- Glutamate

Pyruvate + NADH $\xrightarrow{LDH}$ Lactate + NAD

b) Procedure

Pipette in to test tube	Test
Working ALT reagent	1000µl
Serum/plasma	100µl

Mixed well and aspirated immediately for measurement.

- Blank the analyzer with purified water.
- Read absorbance after 60 sec repeated after every 30 sec i.e. up to 120 sec at 340 nm wavelength.
- Determined the mean absorbance change per min.

c) Clinical Significance

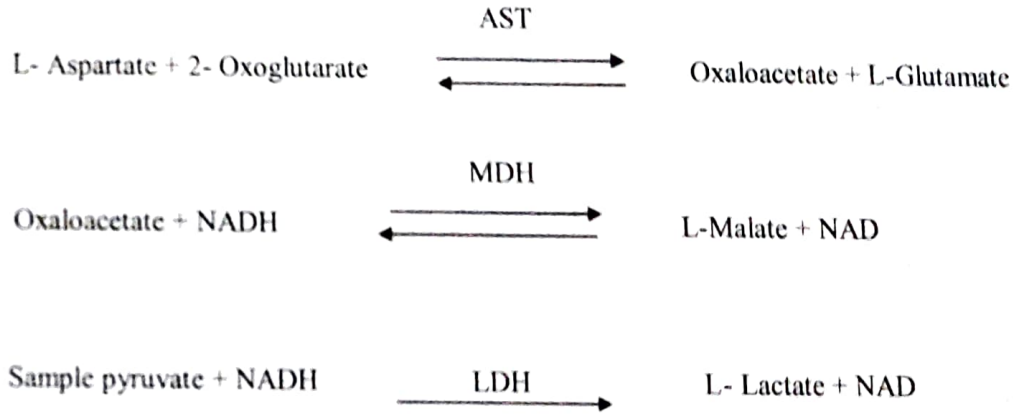
SGPT is present in high concentration in the liver and lesser extent in kidney, heart and pancreas. Increased levels are generally a result of primary liver diseases such as cirrhosis and decreased in

levels may be observed in renal patients.

Estimation of Serum Glutamic oxaloacetic Transaminase (SGOT)

a) Principle:

L-aspartate aminotransferase catalyses the transamination of L- aspartate and 2- oxoglutarate to form L-glutamate and oxaloacetate. In subsequent reaction, Malate dehydrogenase (MDH) reduces oxaloacetate to malate with simultaneous oxidation of nicotinamide adenine dinucleotide to nicotinamide adenine dinucleotide (NAD). The rate of oxidation of NADH is measured kinetically by monitoring the decreases in absorbance at 340 nm and is directly proportional to AST activity in the sample. Lactate dehydrogenase (LDH) is added to enzyme system to prevent endogenous Pyruvate interference, which is normally present in the serum.



b) Procedure

Pipette in to test tube	Test
Working AST reagent	1000µl
Serum/plasma	100µl

Mixed well and aspirated immediately for measurement.

- Blank the analyser with purified water.
- Read absorbance after 30 sec. Repeated after every 30 sec i.e. up to 120 sec at 405 nm wavelength.
- Determined the mean absorbance change per min.

c) Clinical significance

Increased levels are associated with liver diseases or damage, myocardial infarction and decreased levels are observed in patients undergoing renal dialysis.

Estimation of Alkaline Phosphate (ALP) level

a) Principle

Alkaline phosphate in a simple hydrolysis para nitrophenylphosphate into paranitrophenol and phosphate in the presence of magnesium ions. The rate of increase in absorbance of reaction mixture at 405nm due to the liberation of paranitrophenol is proportional to the alkaline Phosphatase activity.

p-Nitro phenyl phosphate + H_2O ⟶ p- Nitro phenol + phosphate

b) Procedure:-

Pipette into test tubes	Test
Buffered substrate (ml)	1.0
Sample(ml)	0.02

- Mix and read absorbance at 30, 60, 90 and 120 seconds at 405 nm. Determine the mean change absorbance per minute and calculate test results.
- Alkaline Phosphatase activity (IU/L) = Δ A/min × factor
- Where factor = 2713 (calculated on the basis of molar extinction coefficient for p-nitrophenol and ratio of total assay volume to the sample volume)

Estimation of Lactase Dehydrogenase (LDH) level

a) Principle:

LDH catalyzes the oxidation of lactate to pyruvate accompanied by the simultaneous reduction of NAD to NADH. LDH activity in serum is proportional to the increase in absorbance due to the reduction of NAD.

Lactate + NAD $\xrightarrow{\text{LDH}}$ Pyruvate + NADH

b) **Clinical significance:**

LDH is an enzyme which present in heart, kidney and other body tissues. It basically increases during myocardial infarction, hepatitis, anaemia, any kidney disease and other muscular injury.

c) Procedure

Pipette into Test Tubes	Test
Working reagent(ml)	1.0
Sample(ml)	0.05

Mix and read first absorbance of the test exactly at one minute and thereafter 30, 60 and 90 sec at 340 nm. Determine the mean change in absorbance per minute and calculate test result.

Estimation of Cholesterol level

a) Principle:

The cholesterol esters are hydrolysed by cholesterol esterase (CE) to give free cholesterol and fatty acids. The free cholesterol then oxidised by cholesterol oxidase to cholesterol-en-3-one with the simultaneous production of hydrogen peroxide. In the presence of hydrogen peroxide, hydrogen peroxides couples with 4-aminoantipyrine (4-AP) and phenol to produce red Quinoneimine dye.

$$\text{Cholesterol esters} \xrightarrow{\text{Cholesterol esterase}} \text{cholesterol + fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{cholest-4-en-3-one} + H_2O$$

$$2\,H_2O_2 + \text{Phenol} + 4 - \text{APP} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + H_2O$$

(4- AAP, 4 – AMINOANTIPYRINE)

b) Procedure

Pipette into tubes marked	Blank	Standard	Test
Serum/ plasma	-	-	10µl
Reagent 2	-	10µ	-
Reagent 1	1000 µl	1000µl	1000µl

Mix well. Incubate at 37°C for 10 minutes or at room temperature (15-30 °C) for 30 minutes.

Programme the analyzer as per assay parameters.

i. Blank the analyser with reagent blank.

ii. Measure absorbance of standard followed by the test.

iii. Calculate results as per given calculation formula.

c) Clinical significance

Serum cholesterol serves an indicator of propensity towards coronary heart disease (CHD), liver and biliary function, intestinal absorption, thyroid function and adrenal disease. Primarily two lipoprotein classes transport the cholesterol. HDL transports cholesterol from tissue to the liver for catabolism while LDL transports cholesterol from sites of origin to deposition in tissues.

Estimation of Triglycerides level

a) Principle:

Triglycerides are hydrolyzed by Lipoprotein Lipase (LPL) to produce glycerol and free fatty acid. In presence of glycerol kinase (GK), adenosine triphosphate (ATP) phosphorylates glycerol to produce glycerol 3- phosphate is further oxidised by glycerol 3- phosphate oxidase to produce dihydroxyacetone phosphate and H_2O_2, in presence of peroxidase, hydrogen peroxide couples with 4- aminoantipyrine (4- AAP) and 4- chlorophenol to produce red quinoneimine dye.

$$\text{Triglyceride} \xrightarrow{\text{LPL}} \text{Glycerol + FFA}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{Glycerol 3- phosphate + ADP}$$

Mg^{+2}

$$\text{Glycerol 3- phosphate} + O_2 \xrightarrow{\text{GPO}} \text{DAP} + H_2O_2$$

$$H_2O_2 + \text{4- AAP} + \text{3, 5-DHBS} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4H_2O$$

b) Procedure

Pipette into tubes marked	Blank	Standard	Test
Serum/ plasma	-	-	10µl
Reagent 2	-	10µ	-
Reagent 1	1000 µl	1000µl	1000µl

Mix well. Incubate at 37°C for 10 minutes or at room temperature (15-30°C) for 30 minutes. Programme the analyzer as per assay parameters.

i. Blank the analyzer with reagent blank.

ii. Measure absorbance of standard followed by the test.

iii. Calculate results as per given calculation formula.

Estimation of Uric Acid level

a) Principle:

Uricase converts uric acid into allantoin and hydrogen peroxide. In presence of peroxidise, hydrogen peroxide oxidatively couples with phenolic chromogens to form a red coloured compound. The concentration of red coloured compound is proportional to the amount of uric acid in sample.

$$\text{Uric acid} + H_2O \xrightarrow{\text{Uricase}} \text{Allantoin} + H_2O_2$$

$$H_2O_2 + \text{Phenolic chromogen} \xrightarrow{\text{Peroxidase}} \text{Red colour compound}$$

b) Procedure

Pipette into tubes marked	Serum / Plasma	Standard	Blank
	0.025 ml	**0.025 ml**	-
Working reagent	**1.0 ml**	**1.0 ml**	**1.0 ml**

Incubate the assay mixture for 5 minutes at 37°C or 10 minutes at room temperature (25-30°C). After completion of the incubation measure the absorbance of assay mixture. Final colour is stable for 30 minutes if not exposed to direct light.

c) Clinical significance

High levels of uric acid linked to impaired renal function and it accelerates the development of hypertension, metabolic syndrome, renal and cardiovascular disease.

Estimation of Total Bilirubin level

a) Principle:

Bilirubin reacts with the diazotised sulphanillic acid to form a colored azobilirubin compound. The unconjugated bilirubin couples with the sulphanillic acid in the presence of caffeine benzoate accelerator. The intensity of the color formed is directly proportional to the amount of bilirubin present in the sample.

Bilirubin + Diazotised sulphanillic acid ⟶ Azobilirubin compound

b) Procedure:

Pipette into clean dry test tubes labeled as Blank (B) and test (T)

Addition Sequence	Blank	Test
Direct Bilirubin Reagent (L1)	1.0 ml	1.0 ml
Direct Nitrate Reagent (L2)	-	0.05 ml
Sample	0.1	0.1

i. Mix well and incubate at room temperature for 10 minutes.

ii. Measure the absorbance at 546 nm of the test sample immediately against their respective blanks.

Estimation of TBARS/MDA activity

a) Principle

Thiobarbituric acid reactive substances-TBARS are formed as a by-product of lipid peroxidation which can detected by TBARS assay using thiobarbituric acid as a reagent. Since reactive oxygen species have tremendously short lives and are difficult to measure directly. Therefore they can be measured as products of the damage produced by the oxidative stress such as TBARS. Assay of TBARS measures malondialdehyde (MDA) present in the sample, as well as MDA generated from the lipid hydroperoxides by the hydrolytic conditions of the reaction.

b) Procedure

The reaction mixture contained 0.1 ml of tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% aqueous solution of Thiobarbituric acid (TBA). The pH of 20% acetic acid was adjusted with 1 N NaOH to 3.5. The mixture was finally made up to 4.0 ml with distilled water and heated at 95°C for 60 min on an oil bath. After cooling under tap water, 1.0 ml of distilled water and 5.0 ml of a mixture of n-butanol and pyridine (15:1 by volume) was added and shaken vigorously on a vortex mixer. After centrifugation at 3000 rpm for 5 min the absorbance of the organic layer (upper layer) was measured immediately at 532 nm using appropriate controls in UV/Visible spectrophotometer (Shimadzu 1700, Singapore) (Ohkawa, Ohishi and Yagi, 1979).

Estimation of Glutathione peroxidase (GPx) activity

a) Procedure:

GPx activity was measured by method described by (Battenberg *et al.*, 1962). Briefly, the reaction mixture contained 0.2 ml 0.4M Phosphate buffer (pH 7), 0.1ml 10Mm sodium azide, 0.2 ml tissue homogenized in 0.4M Phosphate buffer (pH 7), 0.2ml glutathione, and 0.1ml 0.2Mm H_2O_2. The contents were incubated for 10 min at 37° C, 0.4 ml 10% TCA was added to stop the reaction and centrifuged at 3200 rpm for 20min. The supernatant was assayed for glutathione content using Ellman's reagent (19.5g 5, 5'-dithiobisnitrobenzoic acid (DTNB) in

100 ml 0.1% sodium citrate). The activity is to be expressed as μMol of GSH consumed/min/mg protein.

Estimation of GSH activity

a) Principle

GSH is a major non-protein thiol and endogenous antioxidant that counters balance free radical mediated damage. It is involved in the protection of normal cell structure and function by maintaining the redox homeostasis, quenching of free radicals and by participating in detoxification reaction.

b) Procedure

To measure the reduced glutathione (GSH) level, the tissue homogenate (in 0.2 M phosphate buffer pH 7.6) was taken the procedure was followed initially as described by method of(Ellman, 1959). For tissue homogenate, 2.3 ml of potassium phosphate buffer (0.2M, pH7.6) buffer was taken in cell or cuvette, followed by the addition of 0.2ml tissue homogenate. To the tissue homogenate, 0.5ml (DTNB) (0.001M) in a buffer was added. An absorbance of reaction product in the cuvette was read after 5min at 412nm using UV/ Visible double beam spectrophotometer and GSH level was determined from standard curve of reduced GSH obtained with 0.2, 0.4, 0.6, 0.8 and 1nM GSH concentration.

Estimation of Superoxide Dismutase activity

a) Principle:

The method is based on the principle of inhibitory effect of SOD on the reduction of nitroblue tetrazolium (NBT) dye by superoxide radicals, which generated by autoxidation of hydroxylamine. The reduction of NBT is followed by an absorbance increase at 540 nm.

b) Procedure:

To estimate the SOD activity in liver tissue the method described by (Kono, 1978) was followed. The reaction mixture contains 1.3ml of solution A (0.1 m EDTA containing 50 mM Na_2CO_3, pH 10.0), 0.5 ml of solution B (90μm NBT nitroblue tetrazolium dye) and 0.1 ml of solution (0.6%Triton X-100 in solution A), 0.1 ml of solution D (20 mM Hydroxyl amine hydrochloride,

pH 6.0) were mixed and the rate of NBT reduction was recorded for one minute at 560 nm. 0.1 ml of the supernatant was added to the test cuvette as well as reference cuvette, which do not contain solution D. Finally, the percentage inhibition in the rate of reduction of NBT was recorded as described below.

$$\text{Percentage of inhibition of NBT reduction (x)} = \frac{\Delta A/\text{min}_{\text{blank}} - \Delta A/\text{min}_{\text{sample}}}{\Delta A/\text{min}_{\text{blank}}} \times 100$$

The sample volume that produced 50% inhibition was consider as 1 unit of enzyme activity and SOD activity was expressed as unit/g tissue.

Estimation of Catalase activity

a) Principle:

Catalase is the enzyme that breaks hydrogen peroxide into H_2O and O_2. Hydrogen peroxide is often used as a topical disinfectant in wounds and the bubbling that is seen is due to the evolution of O_2 gas. Hydrogen peroxide is a potent oxidizing agent that has break havoc in a cell; because of this any cell that uses oxygen or can live in the presence of oxygen must have a way to get a rid of the peroxide. One of a those way is to make catalase.

b) Procedure:

Catalase activity was determined by the method of (Luck, 1965) wherein the breakdown of hydrogen peroxide (H_2O_2) is measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of H_2O_2, phosphate buffer and 0.05 ml of supernatant of tissue homogenate (10%), and the change in absorbance was recorded at 240 nm. The results were expressed as micromoles of H_2O_2 decomposed per milligram of protein/min

Liver Histopathology

The animals were sacrificed; liver was isolated, washed with ice cold saline and fixed in neutral buffer formalin (NBF) for not less than 6 hours formalin solution (10%).

THESIS OUTLINE:

Chapter 1: Introduction. It covers the aim and objectives of research topic i.e investigating the Role of Pharmacological Intervention in Experimentally Induced Fatty Liver Disease (NAFLD & AFLD).

Chapter 2: Literature Review. It covers the review of work done on fatty liver disease and pharmacological intervention.

Chapter 3: Materials and Methods. It covers the list of material, equipments and methods used in research work.

Chapter 4: Result and Discussion. It covers the Role of Pharmacological Intervention in Experimentally Induced Fatty Liver Disease.

Chapter 5: Summary and Conclusion. It covers the concise statement and key points of pharmacological intervention in fatty liver disease that induced by various inducing models in research work.

Chapter 6: References. It covers every source. The reference list entries contain four main components: author, date, title and source.

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CERTIFICATE
(SYNOPSIS APPROVAL CERTIFICATE)

This is to certify that the Synopsis/Revised Synopsis for Ph.D. Thesis titled, **"Explore the Role of Pharmacological Intervention in Experimentally Induced Fatty Liver Disease"**, submitted by **Ms. Hema Rani** to the Career Point University, Hamirpur (H.P.), India in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in the **Department of Pharmacy , School of Pharmaceutical and health sciences** has been approved by the Research Advisory Committee (RAC) after an Oral Examination of the student on dated 15/5/23.

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